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3,4-Oxo-isopropylidene-shikimic acid promotes adiopkine expression during murine 3T3-L1 fibroblast differentiation into adipocytes

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3,4-Oxo-isopropylidene-shikimic acid;
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Abstract *Objective:* 3,4-Oxo-isopropylidene-shikimic acid (ISA), a derivative of shikimic acid, has exhibited ameliorative effect on cognitive impairment in experimental animal models of dementia. This study investigated the effect of ISA on lipid accumulation and adipokine secretion during differentiation of 3T3-L1 fibroblasts to adipocytes.
Methods: 3T3-L1 cells were cultured and treated with ISA (50–800 μM) from days 3–8. Lipid accumulation and triglyceride content were measured. Gene expression of adipokines (adiponectin, leptin, and resistin), CCAAT/enhancer binding protein (C/EBP) β, C/EBP α and peroxisome proliferator-activated receptor γ (PPAR γ) and PPAR target genes, including adipocyte fatty acid binding protein (aP2) and fatty acid synthase (FAS) were investigated.
Results: ISA promoted 3T3-L1 fibroblast differentiation to adipocytes and increased triglyceride content by 26%. On mechanistic levels, ISA increased expressions of C/EBP β, PPAR γ, C/EBP α, aP2 and FAS. Moreover, ISA stimulated expressions of adipokines secreted by adipocytes, including adiponectin, leptin, and resistin.
Conclusions: These findings demonstrated that ISA promoted adipogenesis by up-regulating expressions of C/EBP β, PPAR γ, C/EBP α, aP2 and FAS, and also stimulated adipokines during

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adipocyte differentiation. Further study should clarify the relationship between stimulation of adipokines and cognitive enhancing effect of ISA.

Introduction

3,4-Oxo-isopropylidene-shikimic acid (ISA, Fig. 1) is a derivative of shikimic acid, which is extracted from star anise (*Illicium verum* Hook. Fil.). Its molecular weight is 214.22 g/mol and it has excellent solubility in water. Our preliminary studies indicated potential therapeutic effect of ISA on cognitive impairment in APP/PS1 double-transgenic Alzheimer mice and focal cerebral ischemic rats.[1–3] ISA also suppressed various experimental thrombosis *in vivo* and *in vitro*.[4] There has been increasing interest in metabolism-related effect on brain function. Retrospective and prospective studies suggest that obesity increases the risk of developing dementia.[5,6] However, little is known about the effect of ISA on this metabolic pathway.

3T3-L1 cell line is one of the most well-researched models for molecular regulation of adipocyte differentiation,[7] and is widely used for studying metabolic syndrome conditions that include obesity and diabetes mellitus. In this study, we investigated the effect of ISA on lipid accumulation and secretion of adipokines including adiponectin, leptin, and resistin during the differentiation of 3T3-L1 preadipocytes into adipocytes.

Materials and methods

Chemicals and reagents

3,4-Oxo-isopropylidene-shikimic acid (ISA, purity > 98%), provided by School of Materia Medica of Beijing University of Chinese Medicine, was dissolved in pure water and kept as a stock solution. Dulbecco's modified eagle medium (DMEM), high glucose-DMEM and fetal bovine serum (FBS) qualified were purchased from Life Technologies (Grand Island, NY, USA). Penicillin and streptomycin mixed solution was obtained from Nacalai Tesque (Kyoto, Japan). Insulin, dexamethasone (DEX), dimethyl sulfoxide (DMSO), 3-isobutyl-1-methylxanthine (IBMX) and triglyceride ELISA kit were obtained from Wako Pure Chemicals (Osaka, Japan). Nitro-blue tetrazolium was purchased from Nacalai Tesque. The RNeasy Mini Kit, RNase-Free DNase Set, QIA shredder were purchased from QIAGEN (Germantown, MD, USA). High capacity cDNA reverse transcription kits were obtained from Applied Biosystems (Tokyo, Japan). The RNase OUT recombinant ribonuclease inhibitor was obtained from Invitrogen (Carlsbad, CA, USA).

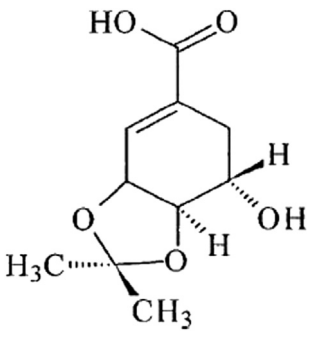

Figure 1 Chemical structure of 3,4-oxo-isopropylidene-shikimic acid (ISA).

Cell culture and treatments

Murine 3T3-L1 preadipocytes (Health Science Research Resources Bank, Osaka, Japan) were grown on Falcon 6-well cell culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in a 5% CO_2 atmosphere at 37°C and maintained in low glucose DMEM (1000 mg D-glucose/L) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The 2-day post-confluent 3T3-L1 cells (designated as Day 0) were incubated with 10% FBS/high-glucose DMEM (HG-DMEM, 4500 mg D-glucose/L) and antibiotics, 500 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin for 3 days (Day 0—2). Cells were then incubated for 2 days in 10% FBS/HG-DMEM with insulin (Day 3—4), and thereafter incubated in 10% FBS/HG-DMEM, which was changed once every 2 days (Day 5—8). Cells receiving ISA were given 10% FBS/HG-DMEM and insulin containing a final concentration of 50, 200, 400, and 800 μM of ISA in diluted water on Day 3 and Day 4. The medium was then changed to 10% FBS/HG-DMEM with ISA for the last 4 days (Day 5—8).

Oil red O staining

On Day 8 of differentiation, 3T3-L1 cells were stained with oil red O to detect droplets in adipocytes. Cells were washed 3 times with PBS, fixed with 4% paraformaldehyde for 30 minutes at room temperature, then washed with 60% isopropanol and dried completely. Thereafter, the fixed cells were stained with 1.8 mg/mL oil red O working solution for 30 minutes at room temperature and then washed twice with 60% isopropanol. Lipid droplets were observed under light microscopy in PBS.

Triglyceride concentration measurement

On Day 8, cells were lysed by RIPA buffer with the protease inhibitor cocktail and SDS and triglyceride content was

measured. The triglyceride concentration was corrected by protein content.

Reverse transcription-polymerase chain reaction

Total RNA was extracted from cultured 3T3-L1 adipocyte (Day 8) using a RNeasy Mini Kit. Complementary DNA was generated from 2 μg of total RNA and synthesized using a high capacity cDNA reverse transcription kit. The PCR conditions were as follows: for glyceraldehydes-3-phosphoate dehydrogenase (GAPDH), 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; for resistin, 27 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; for peroxisome proliferator-activated receptor γ (PPAR γ), adipocyte fatty acid binding protein (aP2), fatty acid synthase (FAS), 28 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; for CCAAT/enhancer binding protein β (C/EBP β), 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; for C/EBP α and leptin, 31 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. To enable comparisons between samples and groups, quantities of all targets in test samples were normalized to GAPDH. The oligonucleotide sequences of all primers are shown in Table 1.

Statistical analysis

All data were presented as mean ± SEM and analyzed by one-way analysis of variance (ANOVA). Multiple group comparisons were made with the least significant difference (LSD) post hoc test using SPSS 17.0 software. Statistical significant difference was defined as a value of $P < 0.05$.

Results

ISA promoted 3T3-L1 preadipocyte differentiation into adipocytes and increased intercellular triglyceride accumulation

Differentiated 3T3-L1 adipocytes began to reserve triglyceride in the cells on Day 4. In this study, the ability of ISA (200, 400 and 800 μM) to induce lipid accumulation was examined by oil red O staining and triglyceride content of 3T3-L1 adipocytes was evaluated on Day 8. The data indicated that adipogenesis was stimulated after addition of ISA 200–800 μM from Day 3 to Day 8. ISA treatment markedly promoted differentiation of preadipocytes into adipocytes. When compared to mature adipocytes, droplets observed in the ISA-treated cells were much more intensive and greater in number (Fig. 2a). Moreover, triglyceride content of adipocytes was significantly increased by 26% after ISA 800 μM treatment ($P < 0.05$) (Fig. 2b).

To evaluate the effect of ISA on adipocyte differentiation, 3T3-L1 preadipocytes were induced to differentiate into mature adipocytes. 3T3-L1 preadipocyte differentiation to adipocytes gradually occurred with lipid accumulation on Day 4. On Day 7, 3T3-L1 cells were differentiated to mature adipocytes and generated oil droplets. During the differentiation process, ISA was added to the medium ata final concentration of 50, 200, 400 and 800 μM from Day 3 to Day 8. On Day 8, 3T3-L1 cells were harvested and lysed. Triglyceride content was then measured by ultraviolet spectrophotometric method according to the manufacturer's protocol.

ISA promoted expressions of transcription factors during 3T3-L1 preadipocyte differentiation into adipocytes

CCAAT/enhancer binding proteins (C/EBP) β was expressed in the early stage of 3T3-L1 differentiation, which was observed within 1 hour after the addition of induction medium and served as the regulator of PPAR γ and C/EBP α. PPAR γ was transcriptionally induced 2 days after induction of differentiation and was at a maximum on Day 3–4, which was necessary and sufficient for adipogenesis *in vivo* and *in vitro*. To detect the effect of ISA on transcription factors, total RNA was purified on Day 8 from differentiated 3T3-L1 cells and underwent PCR analysis. ISA at 200–800 μM significantly increased expressions of C/EBP β (by 30%–60%, $P < 0.01$, $P < 0.001$, $P < 0.001$) and PPAR γ (by 16%–30%, $P < 0.05$, $P < 0.001$, $P < 0.001$). Meanwhile, expression of C/EBP α was significantly increased by 17% after treatment with 400 and 800 μM of ISA ($P < 0.05$) (Fig. 3).

On Day 8, total RNA was extracted from cultured 3T3-L1 adipocytes and preadipocytes using QIAGEN RNeasy mini kit. Complementary DNA was generated from 2 μg of total RNA and synthesized by using high capacity cDNA reverse transcription kit. CCAAT/enhancer-binding protein beta (C/EBP β), C/EBP α and peroxisome proliferator-activated receptor gamma (PPAR γ) were measured by reverse transcription-polymerase chain reaction (RT-PCR) using

Table 1 Primer sequences used for PCR.

Mouse primers	Forward primer	Reverse primer
C/EBP α	ATCCCAGAGGGACTGGAGTT	AAGTCTTAGCCGGAGGAAGC
C/EBP β	GTTTCGGGACTTGATGCAAT	ATGCTCGAAACGGAAAAGG
PPAR γ	TTCAGAAGTGCCTGGCTGTG	TCTTTCCTGTCAAGATCGCC
aP2	AAATCACCGCAGACGACAG	AAATTTCCATCCAGGCCTCT
FAS	CAGTATAAGCCCAAGGCCAA	TAGCCCTCCCGTACACTCAC
leptin	AGAAGATCCCAGGGAGGAAA	TCATTGGCTATCTGCAGCAC
resistin	TCATTTCCCCTCCTTTTCCT	GGCTGCTGTCCAGTCTATCC
GAPDH	GAGGACCAGGTTGTCTCCTG	TGTGAGGGAGATGCTCAGTG

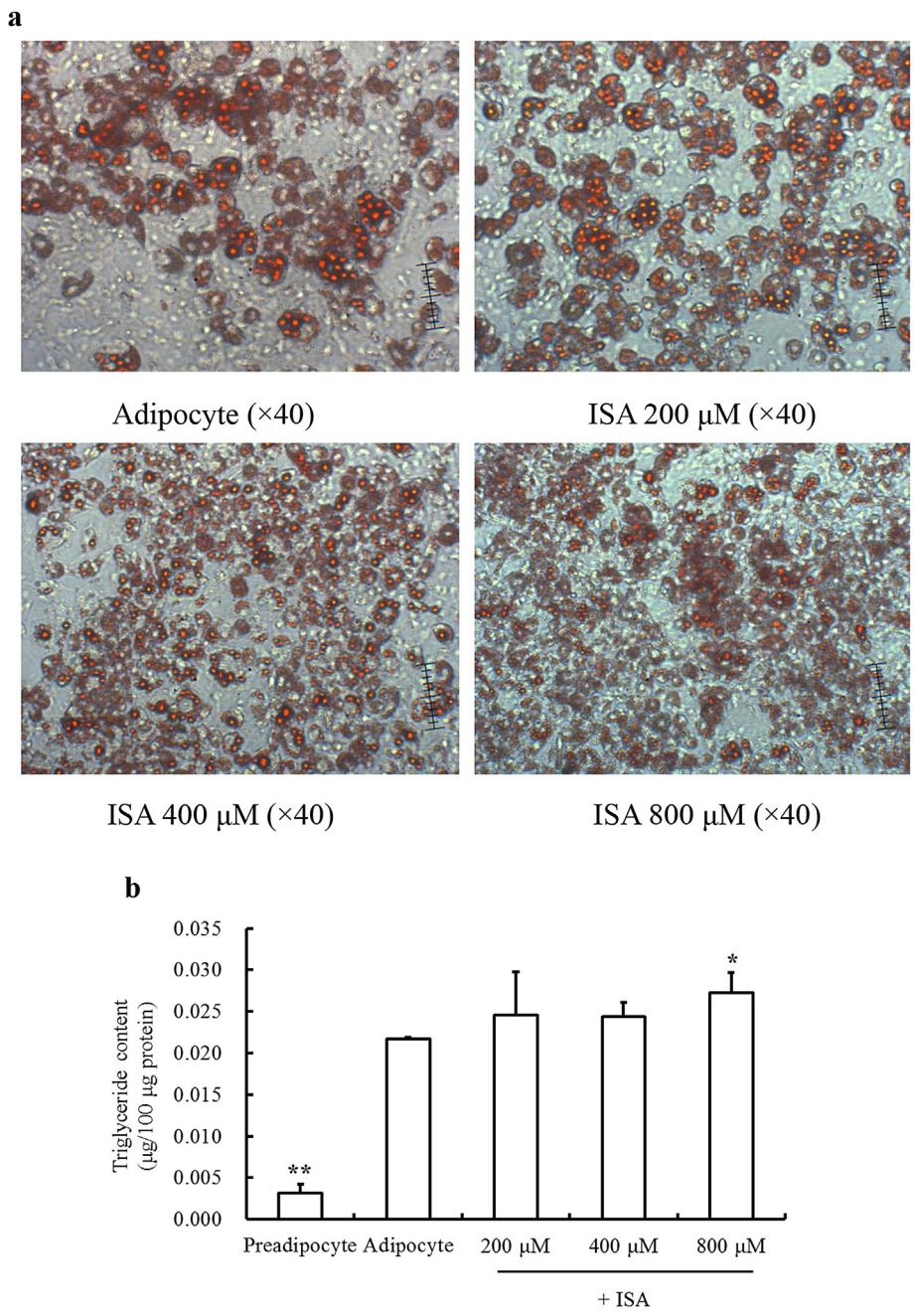

Figure 2 ISA promoted 3T3-L1 preadipocyte differentiation into adipocyte and increased intercellular triglyceride accumulation. a: Oil droplets were stained using oil red O solution. b: Each bar represents mean ± SEM, with $n = 3$. *$P < 0.05$ **$P < 0.001$ vs. adipocyte group, using one-way ANOVA followed by LSD post hoc test.

GAPDH as internal control. Each bar represents mean ± SEM, with $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. adipocyte group, using one-way ANOVA followed by LSD post hoc test.

ISA increased expressions of PPAR target genes including aP2 and FAS during 3T3-L1 preadipocyte differentiation into adipocytes

Peroxisome proliferator-activated receptors (PPARs) are key transcription factors of lipogenesis and adipogenesis. PPARs mediate the transcription of a group of genes related to fatty acid synthesis, transport, storage, and energy expenditure. PPARs target genes including aP2 and FAS were evaluated by PCR. FAS and aP2 were markedly expressed in adipocytes, and interestingly ISA at 50—800 μM significantly increased expressions of aP2 ($P < 0.01$, $P < 0.001$) and FAS ($P < 0.01$, $P < 0.001$) in adipocytes in a dose-dependent manner (Fig. 4).

Adipocyte-specific acid binding protein (aP2) and fatty acid synthase (FAS) mRNA gene expression were tested by RT-PCR method using GAPDH as internal control. Each bar represents mean ± SEM, with $n = 3$. *$P < 0.01$, **$P < 0.001$

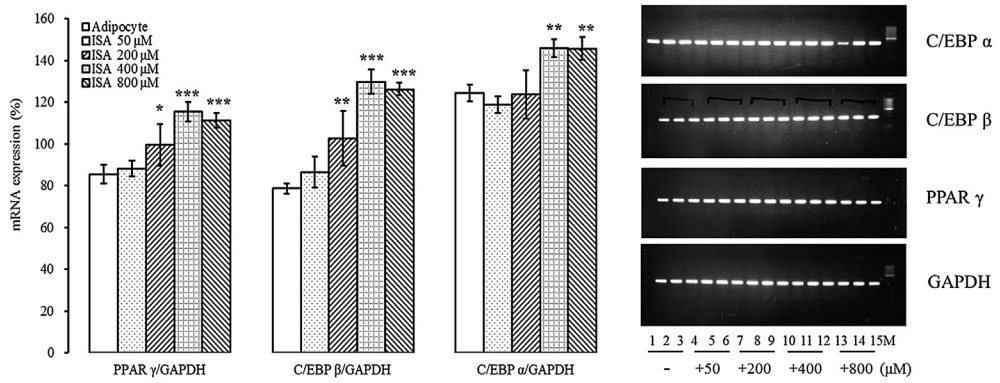

Figure 3 ISA promoted expressions of transcription factors during 3T3-L1 preadipocyte differentiation into adipocytes.

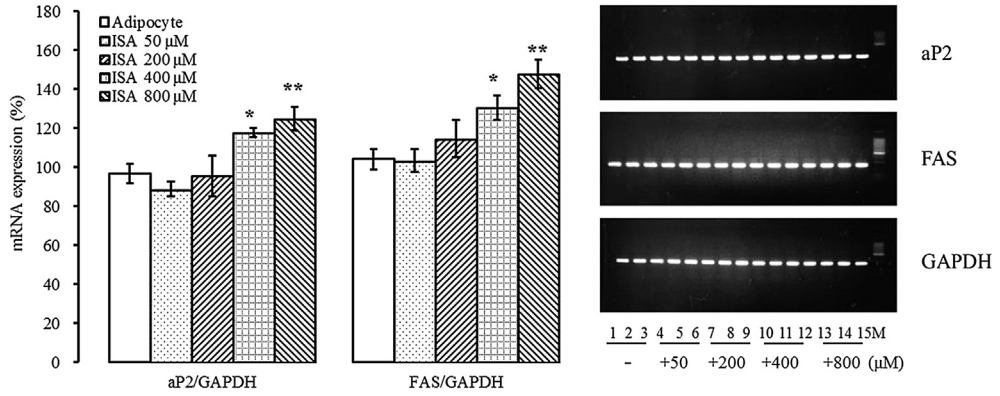

Figure 4 ISA increased expressions of PPAR target genes including aP2 and FAS during 3T3-L1 preadipocyte differentiation into adipocytes.

vs. adipocyte group, using one-way ANOVA followed by LSD post hoc test.

ISA increased adipokinese, including adiponectin, leptin, and resistin during 3T3-L1 preadipocyte differentiation into adipocytes

Adiponectin, leptin, and resistin are known as adipokines, which are mainly secreted by adipose tissue. In this study, genes of adiponectin, leptin, and resistin were highly expressed in adipocytes after addition of induction medium. The adiponectin gene expression was increased significantly after treatment of ISA at 400 and 800 μM by 27% and 37% ($P < 0.001$ and $P < 0.001$), respectively. Meanwhile, gene expressions of leptin and resistin were also increased markedly after addition of ISA at 200—800 μM (for leptin, by 33%—62%, $P < 0.01$, $P < 0.001$; for resistin, by 26%—52%, $P < 0.01$, $P < 0.001$) (Fig. 5).

Adipokines, including adiponectin, leptin, and resistin gene expression were measured by RT-PCR using GAPDH as internal control. Each bar represents mean ± SEM, with $n = 3$. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. adipocyte group, using one-way ANOVA followed by LSD post hoc test.

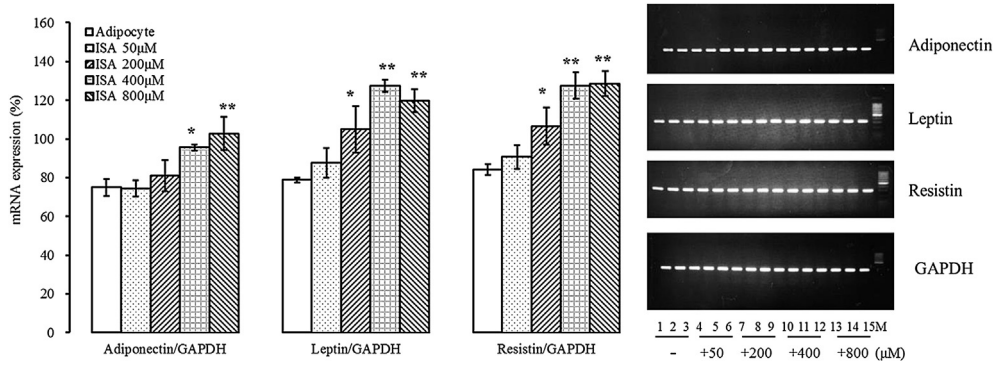

Figure 5 ISA increased adipokines, including adiponectin, leptin and resistin during 3T3-L1 preadipocyte differentiation into adipocytes.

Discussion

3T3-L1 cells are morphologically similar to fibroblastic preadipose cells in the stroma of adipose tissue. After differentiation, they exhibit virtually all of the characteristics associated with adipocytes present within the adipose tissue.[8] After treatment with the differentiation cocktail (insulin, DEX, IBMX), 3T3-L1 preadipocytes undergo differentiation and progress to mature adipocytes. Members of C/EBP family (C/EBP β and C/EBP α) and PPAR γ play essential roles in this differentiation process. C/EBP β expresses early in this process, which is a prerequisite for mitotic clonal expansion,[9] and activates transcription of the C/EBP α and PPAR γ. PPAR γ and C/EBP α alone or in cooperation with each other induce the transcription of many adipocyte genes encoding the proteins and enzymes, adipocyte P2 gene (aP2) and fatty acid synthase (FAS), which are the late key markers for maintaining the phenotype of adipocytes. Among these, aP2 is considered to be involved in fatty acid transport, storage, and export, and FAS is a key factor of adipogenesis and lipogenesis.[10]

Oil droplet accumulation and triglyceride deposition in 3T3-L1 cells were significantly increased after treatment with ISA, which was the first evidence to demonstrate the stimulation effects of ISA on triglyceride deposition. These results indicated that ISA could promote adipocyte differentiation and adipogenesis in the 3T3-L1 cell line. On mechanistic levels, ISA significantly promoted expressions of C/EBP β, C/EBP α, and PPAR γ, as well as aP2 and FAS, which lead to the differentiation of adipocytes.

Over the last two decades, increasing studies have focused on the correlation of high-fat diet and cognition. Adipokines including leptin, adiponectin, and resistin produced by adipose tissue, are able to cross the blood—brain barrier and may be implicated in cognition deficits.[6,11] Specifically, leptin, a pro-inflammatory adipokine, has been shown to promote memory, including enhancing long-term potentiation with consequent cognitive improvement and also to influence hippocampal synaptic plasticity.[12,13] Increasing leptin availability is considered a valid target for a novel treatment for Alzheimer disease, a hypothesis that has been confirmed by mechanistic and animal study as well as ample clinical data.[14,15] Adiponectin, an anti-inflammatory and insulin-sensitizing compound, also exerts neuroprotective effect in addition to its anti-angiogenic, anti-atherogenic, and vasodilatory actions.[16—19] Evidence has also demonstrated that resistin has neuroprotective effects by inhibiting oxidative stress and apoptosis.[20]

Previously, we focused mainly on the cognitive enhancing effect of ISA, with preliminary results demonstrating that ISA appears to improve learning and memory in APP/PS1 double-transgenic Alzheimer mice and focal cerebral ischemic rats. In this study, the results showed that ISA stimulated expressions of adiponectin, leptin, and resistin secreted by adipocytes. As described above, up-regulating of adipokines in adipocytes of ISA might be implicated in cognitive enhancement of ISA. However, further confirmatory investigation is needed.

Acknowledgments

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